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TITLE: Studying the Roles of GRK2-Mediated Smad2/3 Phosphorylation as a Negative Feedback Mechanism of TGF-Beta Signaling and a Target of Breast Cancer Therapeutics

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14. ABSTRACT

The canonical TGFb/Smad signaling axis promotes breast cancer metastasis, as blocking this pathway could slow down metastasis in animal models. Since Smad2 and Smad3 are transcription factors, they are not ideal drug targets. As such, investigating intracellular signaling mechanisms that regulate Smad activity is highly meaningful not only for understanding TGFb's pro-invasive functions in breast cancer but also for identifying new leads to design therapies that block TGFb signaling in metastatic breast cancer.

We identify two of such mechanisms, mediated through cell signaling molecules GRK2 and BCAR3, which could antagonize TGFb signaling in human breast cancer cells. During the tenure of the traineeship, we performed biochemical studies to elucidate how these signaling molecules could block TGFb/Smad signaling; and performed cell-based functional analysis to determine whether these molecules could modulate TGFb's pro-invasive functions. We found that both GRK2 and BCAR3 were potent inhibitory molecules of Smad activation. They both antagonize TGFb-mediated gene transcription and TGFb-induced breast cancer cell invasion. High expression of GRK2, or BCAR3, associates with lower chance of relapse and distant metastasis among breast cancer patients. As such, mimicking GRK2 or BCAR3's function in breast cancer cells could likely decrease the invasive properties of these cells.

15. SUBJECT TERMS
TGFb, Smad, breast cancer, invasion, GRK2, BCAR3

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1. Introduction:

During breast cancer progression, alongside the development of hormone-independent growth mechanisms, cancer cells have been shown to alter their biological response to the transforming growth factor β (TGFβ) [1]. TGFβ family growth factors, through inducing cell cycle arrest and apoptosis, inhibit proliferation in the mammary epithelium and in differentiated, early stage breast tumors [2-5]. These functions are lost and replaced by tumor promoting and pro-metastatic responses in poorly-differentiated, advanced stage breast tumors [6, 7] In cancer cells representing such tumors, TGFβ transcriptionally reprograms cells to induce epithelial to mesenchymal transition (EMT), cell migration and invasion[8, 9]. In addition, in the stroma, TGFβ promotes local and systematic immune suppression, thereby allowing transformed cells to escape immune surveillance, further promoting tumor metastasis [8, 10, 11] Most of the above mentioned biological functions of TGFβ are attributed to a canonical signaling pathway mediated by the Smad transcription factors [12]. TGFβ binding to its receptors (type I and type II serine threonine kinases) leads to the recruitment and phosphorylation of Smad2/3 and their association with Smad4. The activated Smads then collectively translocate into the nucleus where they bind to regulatory elements on the promoter regions of their target genes to regulate gene transcription[13]. The canonical TGFβ/Smad signaling axis is central to TGFβ-mediated breast cancer cell migration and tumor metastasis. Altering the function of key components of the TGFβ/Smad signaling, using RNA interference or decoy ligand traps approaches, impairs formation of breast cancer metastasis in experimental models[6, 14, 15]. As such, understanding how intracellular mechanisms regulate Smad signaling provides insights into the biology of metastatic breast cancer and novel means of treatment and prognosis.

During the tenure of the traineeship, I studied two of such mechanisms, namely those mediated through G Protein Coupled Receptor Kinase 2 (GRK2) and Breast Cancer Anti-Estrogen Resistance 3 (BCAR3). During the first two years of the study, we conducted biochemical analysis to investigate how these two signaling molecules could antagonize TGFβ/Smad signaling. They behave similarly, by antagonizing C-terminal serine phosphorylation on the Smads, thereby blocking Smad activation. However, they do so through different mechanisms. Whereas GRK2 phosphorylates a defined serine/threonine residue on the linker region of the Smad, BCAR3 recruits another signaling molecule, p130Cas, which physically interacts with the C-terminal of the Smads. In the last year of the traineeship, I conducted cell-based biological assays to evaluate whether GRK2 and BCAR3 could modulate TGFβ's pro-invasive responses in breast cancer cells. We find that these factors, when overexpressed, remarkably block TGFβ-mediated cell invasion. While we have technical difficulty to stably transfect these factors into breast cancer cell lines in order to test their effects on invasion *in vivo*, we have changed strategy to adopt a virus infection-based method to generate inducible stably cell lines.

2. Body:

Task 1: Generating stable cell lines expressing ectopic GRK2 and BCAR3.

According to modified SOW, we stably transfected SUM-159 cells and SCP2 cells with GRK2 or BCAR3 expression vectors to select for stable cell lines. We are able to obtain stable expression of FLAG-tagged GRK2 and BCAR3, but at low expression level. We further tested whether these stable cells carry impaired response to TGFβ. Stable

expression of GRK2 and BCAR3 has modest effects on decreasing TGFβ-induced transcription from 12CAGA-lux, a Smad-responsive artificial reporter gene (data not shown). This is likely due to the low level of expression of these factors.
To obtain cell lines that allow us to conduct *in vivo* experiments, we have constructed viral vectors for inducible expression of GRK2 and BCAR3 in human breast cancer cells. To do so we have subcloned Flag-tagged human GRK2 or BCAR3 open reading frames into pLVX-TRE3G lentiviral vector, produced viral particles, infected SUM-159 cells and recently established pools of cells resistance to antibiotic selection. We are currently validating inducible expression of GRK2 or BCAR3 by Western blot and Immunofluorescence.

Task 2: Evaluate the effects of BCAR3 on Smad signaling and TGFβ-induced cell invasion.
In previous funding years, we found that BCAR3 is an endogenous factor in breast cancer cells that antagonizes Smad activation. We further tested whether overexpressing BCAR3 could impair Smad-mediated transcriptional responses. We found that BCAR3 overexpression could effectively block TGFβ-induced Smad nuclear translocation and Smad-dependent transcription (please see previous reports and manuscript/Appendix A).
TGFβ induces cell migration and invasion in basal-like metastastatic breast cancer cells, such as MDA-MB-231 cells and SCP2 cells. We opted for a real time live cell imaging approach and found that knocking down endogenous BCAR3 in SCP2 cells promoted TGFβ-induced cell migration (please see attached manuscript). Moreover, knocking down BCAR3 in SCP2 cells resulted in remarkable increases in TGFβ-induced cell invasion through matrigel (Figure 1/Appendix B). We also obtained similar results using another basal-like breast cancer cell line, BT-549 cells. These results suggest that endogenous BCAR3 is an antagonistic mechanism of TGFβ's pro-invasive responses.
In addition to the transwell experiments proposed in the SOW, we also used a gelatin matrix degradation assay to study cell invasion, as this method allows us to visualize single cell invasion and morphology of invadopodia. In MDA-MB-231 cells, TGFβ remolded invadopodia structure, from a scattered pattern to a bundled pattern that favors cell invasion. Knocking down BCAR3 facilitated this response, and increased both invadopodia number and total area of digestion in response to TGFβ (please see manuscript/Appendix A).

Task 3. Evaluate the effects of ectopic GRK2 in antagonizing metastasis *in vivo*.
As discussed in Task 1, we experienced technical difficulty generating stable cell lines that could express ideal levels of GRK2 for *in vivo* studies. To conduct the proposed *in vivo* studies, we are currently selecting viral-infected SUM-159 cells for inducible GRK2 expression. At the main time, we addressed this task using the matrix degradation assay as described in Task 2. We transfected FLAG-tagged GRK2 into MDA-MB-231 cells, allowed cells to invade into gelatin matrix for 48 hours, and then observe the effect of ectopic GRK2 on matrix degradation. Under these conditions, we observed that under each cell there were black dots joining into large areas, representing degradation of fluorescence gelatin matrix. However, this effect seemed to be absent in cells expressing FLAG-tagged GRK2 (Figure 2/Appendix B). Furthermore, we also found that ectopic GRK2 expression in MDA-MB-231 cells could block TGFβ-induced matrix degradation

(Figure 2/Appendix B). This result serves as a proof-of-principle for our proposed *in vivo* experiments, which we will conduct as soon as we obtain the stable inducible cells.

Task 4. Training.
With the support of the traineeship, I have attended several courses, training workshops and conferences, as listed in previous annual reports. During the past year, I have attended a symposium hosted by NIH's Integrative Network-Based Cellular Signature program and a symposium hosted by the Cancer Center of Beth Israel Deaconess Medical Center, in Boston MA and on consecutive dates. These events extend my scope of knowledge on Systems Biology and Cancer Biology, respectively. In particular, they further shaped my research interest on breast cancer, to studying mechanisms that influence drug responses and prognosis using genomic and proteomic approaches.
During the tenure of the traineeship, I prepared a first author manuscript reporting the novel role of BCAR3 as an antagonistic factor of Smad signaling. It is currently under peer review. I also contributed to a few manuscripts; two of which are published and one is under peer review (Appendix C).

3. Key Research Accomplishments:
- Characterize clinical implications of GRK2 and BCAR3 in human breast cancer.
- Characterize Smad mutants as novel inhibitors of Smad signaling.
- Identify BCAR3 to be a novel inhibitor of Smad signaling.
- Evaluate BCAR3's role in antagonizing TGFβ's pro-invasive responses.

4. Reportable Outcomes:
- Manuscripts: one first author manuscript in submission and four co-author manuscripts
- Presentations: three conference poster presentations (Cold Spring Harbor Symposium and AACR conferences) and several oral presentations in local and international conferences
- Training and workshops: Welcome Trust Advanced Course, Confocal Microscopy training course, Beth Israel Deaconess Medical Center Co-clinical workshop, and a series of other workshops and short courses.
- Degree: currently writing my dissertation
- Cell lines: SUM-159 cells overexpressing FLAG-tagged BCAR3 and GRK2; and SCP2 cells overexpressing Smad mutants

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6. Appendix:

A. Manuscript entitled "Breast Cancer Anti-Estrogen Resistance-3 (BCAR3) inhibits TGFbeta/Smad signaling and associates with favorable breast cancer disease outcomes".
B. Figure 1 and Figure 2
C. Publications and posters during the tenure of the traineeship

Appendix A:

Breast Cancer Anti-Estrogen Resistance-3 (BCAR3) inhibits TGFβ/Smad signaling and associates with favorable breast cancer disease outcomes

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Abstract:

In this study, we investigated the role of the Breast Cancer Anti-Estrogen Resistance 3 (BCAR3) gene in breast cancer progression. Our results define a novel function for BCAR3 as a potent inhibitor of the TGFβ signaling pathway. We found BCAR3 to inhibit Smad activation, Smad-mediated gene transcription, Smad-dependent cell migration and matrix digestion in breast cancer cells. Furthermore, we found BCAR3 gene expression itself to be repressed by TGFβ, defining a novel positive feedback loop mechanism downstream of the TGFβ/Smad signaling pathway. Examination of BCAR3 expression levels in large cohorts of breast cancer patients also indicate that BCAR3 bears a true prognostic value in human breast cancer. Indeed, loss of BCAR3 expression in primary breast tumors correlates with distant metastasis free survival (DMFS) and relapse free survival (RFS) poor outcomes, regardless of molecular subtype and treatment. We also found a strong correlation between the loss of BCAR3 expression and lymph node invasion in human breast cancer, further suggesting a role for BCAR3 in preventing disease progression. Traditionally, BCAR3 has been considered to be associated with aggressive breast cancer phenotypes. However, our results indicate that BCAR3 acts as a putative suppressor of breast cancer progression by inhibiting the pro-metastatic TGFβ/Smad signaling pathway in invasive breast tumors. These data provides new insights into BCAR3's molecular mechanism of action and highlight BCAR3 as a novel TGFβ/Smad antagonist in breast cancer.

Keywords: BCAR3, TGFβ, Smad, migration, invasion, breast cancer prognosis

Introduction:

Breast tumorigenesis and progression are controlled by multiple hormone/growth factor/cytokine signaling pathways, which are ideal therapeutic targets. Targeted therapies against breast cancer, such as those developed towards the Estrogen Receptor alpha (ERα) or the Her2 receptor tyrosine kinase, have shown some levels of success[1,2]. However, clinical observations also indicate that tumors which initially respond to targeted therapies often relapse and acquire resistance to the treatments[3,4]. Several genes, collectively named Breast Cancer Anti-Estrogen Resistance (BCAR) genes were found to induce estrogen-independent cell growth in estrogen-dependent breast cancer cells[5]. Two members, BCAR1/p130Cas and BCAR3 are found to form a complex by directly interacting with each other[6,7]. Individual overexpression of these genes allows estrogen-dependent breast cancer cells to proliferate under the presence of Tamoxifen [5,8]. Ectopic overexpression of BCAR3 in breast cancer cells activate Src and FAK kinases, leading to p130Cas tyrosine phosphorylation and increased cell attachment to fibronectin and cell motility[7,9]. Therefore, BCAR3 is traditionally considered to play a role in mediating aggressive breast cancer phenotype. However, a previous report suggested BCAR3 expression to correlate with favorable outcome in progression free survival in a cohort of estrogen receptor positive breast cancer patients who had received Tamoxifen[10]. As such, the regulatory role played by BCAR3 in breast cancer cells remained unclear and controversial and is aimed to be addressed in this study.

During breast cancer progression, alongside the development of hormone-independent growth mechanisms, cancer cells have been shown to alter their biological response to the transforming growth factor β (TGFβ) [11]. TGFβ family growth factors, through inducing

cell cycle arrest and apoptosis, inhibit proliferation in the mammary epithelium and in well-differentiated, early stage breast tumors [12-15]. These functions are lost and replaced by tumor promoting and pro-metastatic responses in poorly-differentiated, advanced stage breast tumors [16,17,18,19]. In cancer cells representing such tumors, TGFβ transcriptionally reprograms cells to induce epithelial to mesenchymal transition (EMT), cell migration and invasion[19,2019]. In addition, in the stroma, TGFβ promotes local and systematic immune suppression, thereby allowing transformed cells to escape immune surveillance, further promoting tumor metastasis [19,21,22]. Most of the above mentioned biological functions of TGFβ are attributed to a canonical signaling pathway mediated by the Smad transcription factors [23]. TGFβ binding to its receptors (type I and type II serine threonine kinases) leads to the recruitment and phosphorylation of Smad2/3 and their association with Smad4. The activated Smads then collectively translocate into the nucleus where they bind to regulatory elements on the promoter regions of their target genes to regulate gene transcription[24]. The canonical TGFβ/Smad signaling axis is central to TGFβ-mediated breast cancer cell migration and tumor metastasis. Altering the function of key components of the TGFβ/Smad signaling, using RNA interference or decoy ligand traps approaches, impairs formation of breast cancer metastasis in experimental models[16,25,26]. As such, understanding how intracellular mechanisms regulate Smad signaling provides insights into the biology of metastatic breast cancer and novel means of treatment and prognosis.

In this study, we define a novel regulatory pathway directly linking the TGFβ/Smad signaling axis to BCAR3. Our data highlight BCAR3 as a potent inhibitor of the TGFβ/Smad signaling pathway. We found BCAR3 to promote an interaction between

Smad2/3 and p130Cas, leading to inhibition of Smad activation, Smad-mediated gene transcription and Smad-dependent cell migration in breast cancer cells. Interestingly, we also found BCAR3 expression to be controlled by TGFβ itself, as TGFβ treatment decreases BCAR3 expression in a Smad-dependent manner. This define a novel positive regulatory feedback loop, through which TGFβ signaling further induces its effects by blocking expression of the Smad inhibitor, BCAR3. We also report a true prognostic value of BCAR3 in human breast cancer. We found that loss of BCAR3 expression in primary breast tumors correlates with poor outcomes, regardless of molecular subtype and treatment. Taken together, our study indicates that BCAR3 is a novel antagonist of TGFβ functions in breast cancer cells, and loss of BCAR3 function correlates with poor outcomes in breast cancer patients.

Methods:

Gene expression analysis: Using GOBO, patient outcomes were quarried for 10 years. Compiled cohorts of patients were divided as described in the text, based on reading from an Affymetrix BCAR3 probe on microarrays (204032_at). In the analysis of endocrine treated patients, log rank readings from the probe targeting BCAR3 were extracted from the NCBI-GEO dataset GDS807, and plotted.

Cell culture: MCF-7, MDA-MB-231 and BT-549 cells were obtained from ATCC and maintained in DMEM (Hyclone) supplemented with 10% FBS. Inducible BCAR3 MCF-7 cells were maintained in DMEM supplemented with 10% FBS, 100μg/ml G418 and 1μg/ml Puromycin. SUM-149PT and SUM-159PT cells were obtained from Dr. Stephen Ethier, and were maintained in F-12 nutrient mixture (Hyclone) supplemented with 5%

FBS, 5μg/ml insulin and 1μg/ml hydrocortisone. SCP2 cells were obtained from Dr. Joan Massague.

Constructs and Transfection: BCAR3 siRNAs (catalogue number SASI_Hs01_00236261 and SASI_Hs02_00335873), p130Cas siRNA (SASI_Hs02_00345830) and scrambled control siRNA were manufactured by Simga-Aldrich. In the Smad phosphorylation experiments, the scrambled control siRNA was manufactured by Ambion (Ambion scrambled control #2). Dr. Laurence Quilliam kindly provided the FLAG-tagged mouse AND-34 expression vector. The GST-tagged rat p130Cas expression vector was obtained from Addgen (catalogue number 15001). Transfections were carried out using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions.

SDS-PAGE and Western blot: Cells were lysed with RIPA buffer containing 1% Triton-X, protease inhibitors and phosphatase inhibitors. Total protein lysates were quantified and 50 μg lysates were separated by SDS-PAGE followed by transferring onto nitrocellulous membrane and subjected for Western blot as previously described. To obtain nuclear extracts, cells were lysed with PBS containing 1% NP-40. The nucleus were washed in the lysis buffer for multiple times and lysed with loading dye containing SDS, as described in a protocol described by the others.

RNA extraction, reverse transcription and real-time PCR: Total RNAs were extracted with Trizol reagent (Life Technologies) following the manufacturer's instructions. RNA samples were reverse-transcribed using MML-V (Life Technologies) and subjected for real-time PCR, with ribosomal 18S RNA as internal control. PCR reactions were carried

out using Ssofast Evergreen super mix (Bio-rad) following the manufacturer's instructions

Cell viability assay: Inducible MCF-7 cells were plated into 96-well plates (5,000 cells/well), and cultured in complete DMEM with or without doxycycline for 96 hours. Cells were then serum starved with or without doxycycline. Under each condition, paired wells of cells were treated with or without 200pM TGFβ for 72 hours. Cells were then incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 hours, gently washed with PBS. Each well received 200µl DMSO to suspend converted formazan, subjected for absorption reading at 570nm.

Confocal microscopy: Cells transfected with FLAG-AND-34 were seeded on cover slips, treated as described in figure legends, fixed with 3.7% paraformaldehyde in PBS for 15 minutes, and then permeabilized with 0.1% triton-X in PBS for 3 minutes. After blocking for 1 hour at room temperature in 2% BSA in PBS, cells were co-stained with a mouse anti-FLAG antibody (#M2, Sigma, 1:500) and a rabbit anti-phospho-Smad3 antibody (#9520, Cell Signaling, 1:500), or Alexa568-labled Phalloidin (Life Technologies). Cells were then stained with Alexa488-labled goat-anti-mouse secondary antibody (Life Technologies, 1:500) and Alexa568-labled goat-anti-rabbit secondary antibody (Life Technologies, 1:500), except in the case when Phalloidin used. Following DAPI counterstaining and mounting, images were taken using a X63 immersion oil objective on an LSM780 confocal microscope (Carl Zeiss). Images were taken in a multi-track scanning mode at a 1024X1024 resolution. Excitation wavelengths were set at 490nM (Argon laser) and 570nM (HeNe laser) for detecting emission wavelengths at around 520nM (for Alexa-488) and around 600nM (for Alexa-568), respectively. Images were

converted to 16-bit TIFF RGB format by ImageJ. Images of individual channels were converted to grey scale, and merged images were set at auto-contrast using Photoshop CS6. Quantifications of phospho-Smad levels were performed using ImageJ software (5 images per condition).

Migration Assay: Scratch-based migration assays were carried out with an Incucyte automatic system (Essen Bioscience) according to the manufacturer's protocol. Briefly, cells transfected with siRNA were seeded onto I96-well ImageLock plate one day post-transfection at a density of 50,000 cells per well. Cells were then starved overnight. Monolayers of cells were scratched using a scratching apparatus that produced highly identical scratches in each well. Cells were then treated with 100pM TGFβ. The Incucyte system was programmed to obtain real-time phase contrast image of the wounded at 12 time points. In the BCAR3 siRNA experiments, images were taken every 4 hours for 48 hours. In the double knocking-down experiments, images were taken every 3 hours for 36 hours. At least 3 independent experiments were performed, and each experiment contains 4 biological replicates for every condition. Results from one representative experiment were shown. Cell migration was quantified and expressed as Relative Wound Density, which indicates the ratio of sharpness of the wounded area and of adjacent non-wounded area.

Gelatin digestion assay: Cover slips were first treated with poly-D-lysine and glutaraldehyde, then coated with 0.1% pig gelatin solution containing Alexa488-conjugated gelatin for 3 hours. The slides were quenched with 0.1% sodium borohydride solution prior to be seeded with 100,000 cells. Six hours after seeding, the cells were starved and treated with or without 200pM TGFβ for 36 hours. Fixing, staining and

imaging procedures are described above in the Confocal Microscopy section. Images show representative images of 3 independent experiments. Quantifications show averages and standard deviations of 10 cells on 7-10 LSM image files of a representative experiment.

Results:

1. BCAR3 expression correlates with favorable breast cancer disease outcome.

BCAR3 is considered to be associated with aggressive disease phenotypes as it promotes estrogen-independent cell proliferation, cell migration, and cell-ECM contacts[5,7,9,27,28]. However, a clinical study suggests BCAR3 expression to be a single factor to predict favorable progression-free survival of patients who received Tamoxifen treatment[10]. To investigate BCAR3's clinical implications, we used Gene Expression-Based Outcome for Breast Cancer Online (GOBO) [29] to generate Kaplan-Meier survival curves of breast cancer patients from published microarray datasets in the NCBI Genome Omnibus. Consistent with the above mentioned study[10], we found some level of correlation ($p=0.09$, data not shown) between high BCAR3 expression and low rate of disease progression in Tamoxifen-treated patient. More importantly, we extended our study to examine progression-free survival (PFS) to two distinct cohorts of patients: a compiled cohort that underwent various treatment plans and a true-prognostic cohort that received no systematic therapy. As shown in Fig.1A, in the compiled cohort, we found that those with low BCAR3 expression (grey) had significantly worse prognosis compared to patients with high BCAR3 levels (red), indicating that BCAR3 expression favors progression-free survival for patients who received various treatment plans. We also found a similar trend in non-treated patient cohort (Fig. 1B). This not only demonstrates a true prognostic

value for BCAR3, but also implicates that loss of BCAR3 expression may be involved in breast cancer progression. We further analyzed and correlated BCAR3 expression levels with distant metastasis or disease relapses. Consistently, we found BCAR3 expression to positively correlate with higher distant metastasis free survival (DMFS) in both an overall cohort and a non-treated cohort of patients (Fig. 1C and 1D, respectively). Furthermore, BCAR3 expression also correlated with higher relapse free survival (RFS) in the overall cohort (Fig. 1E). In the non-treated cohort, when patients are separated by median expression level, no significant link is observed between BCAR3 and RFS (data not shown). However, when patients are separated into more refined groups, we found that the group that expressed the lowest level of BCAR3 had significant worse prognosis, whereas risks of the 4 other groups were comparable (Fig. 1F). This suggests a dose-dependent like effect of BCAR3, whereas a slight decrease of BCAR3 expression may not be detrimental to relapse-free survival, a severe loss of BCAR3 expression correlates with markedly increased tumor relapse.

Complimentary to these findings, we further investigated whether established disease characteristics can be traced backwards to BCAR3 expression levels in primary breast tumors. We analyzed both disease relapse and lymph node positivity, as they represent two critical indicators of the aggressiveness of the disease. To do so, we grouped patients based on established outcomes and surveyed for difference in BCAR3 expression between groups. In a cohort of patients with ER+ breast tumors treated with endocrine therapy for 5 years (NCBI-GEO GDS807)[30], those who developed disease relapse had lower levels of BCAR3 expression in their primary tumors (Figure 1G) and lower BCAR3 transcript abundance (data not shown). These results, together with Kaplan-

Meier analysis, highlight a high degree of overlapping between patients with low BCAR3 levels and patients with disease relapse. Additionally, using the ROCK breast cancer functional genomics database[31], from a CGH dataset designed to identify copy number abnormalities in breast cancer[32], we clearly established a correlation between advanced Tumor N-stage/lymph node status and loss of heterozygosity at BCAR3 alleles in breast cancer patients,. Particularly, combined loss and deletion of BCAR3 alleles increased from 18% in N0 tumors (no lymph node invasion), to 31% in N1 tumors (tumor cells in regional lymph nodes), and to 50% in N2 tumors (tumor cells in regional and distant lymph nodes). Altogether, these results indicate that loss of BCAR3 expression correlates with an invasive tumor phenotype, increased lymph node involvement. Concomitantly, high BCAR3 expression levels are predictive of significantly improved progression-free, distant metastasis free and relapse free survivals. Our data also suggest that BCAR3 likely plays a tumor suppressor-like role by preventing disease progression in breast cancer patients, independently of treatments received.

2. BCAR3 antagonizes TGFβ-induced Smad phosphorylation and nuclear translocation.

We then sought to investigate the molecular mechanisms by which BCAR3 exerts this protective role in breast cancer cells. Interestingly, a BCAR3-interacting protein, p130Cas, was previously shown to directly interact with Smad2/3, thereby blocking Smad C-terminal serine phosphorylation and activation, resulting in an inhibition of TGFβ signaling [33,34]. As the TGFβ/Smad signaling pathway has been shown to play a prominent role in breast cancer progression and tumor metastasis, we investigated whether BCAR3 could regulate TGFβ/Smad signal transduction. We initially examined the relative protein expression levels of BCAR3 and p130Cas in a panel of breast cancer

cell lines representing different molecular subtypes and phenotypes of breast tumors. Similar to previously reported findings[35], we found that BCAR3 expression level was relatively high in estrogen-independent breast cancer cells (Fig.2A). Additionally, BCAR3 expression generally correlated with breast cancer subtype. Luminal-like MCF-7 and SK-BR-3 cells express relatively low levels of BCAR3, whereas basal-like MDA-MB-231, SCP2, BT-549 and SUM-149PT cells expressed relatively high levels of BCAR3 (Fig.2A). However, SUM-159PT cells, which are ER- and estrogen-independent in culture and as xenograft, also expressed a relatively low level of BCAR3 (Fig. 2A). This may likely be due to the anaplastic nature of the origin of these cells[36]. Furthermore, p130Cas is fairly abundant in all cells tested, and its expression did not seem to correlate with either ER status or cancer subtype (Fig.2A). These results suggest that while p130Cas expression maybe a universal event in most types of breast cancer cells and tumors, high BCAR3 expression is likely specific in cells of a basal like breast cancer phenotype.

We used both loss-of-function and gain-of-function approaches to investigate whether BCAR3 is involved in regulating the TGFβ/Smad signaling axis in breast cancer cells. SCP2 cells were derived as a sub-progeny of MDA-MB-231 cells, and are highly invasive and highly responsive to TGFβ[26,37]. Moreover, these cells express high levels of BCAR3 among the panel of breast cancer cell lines that we tested (Fig.2A). In SCP2 cells, TGFβ induced Smad3 phosphorylation in time dependent manner, however, this effect was markedly potentiated when BCAR3 gene expression was silenced, using a specific siRNA, suggesting that BCAR3 is inhibitory to TGFβ and Smad signaling (Fig.2B) .

To further address this inhibitory role, we examined the effect of ectopic BCAR3 on Smad signaling in MCF7 cells, which express fairly low or undetectable endogenous BCAR3 levels. We used an inducible stable MCF-7 cell line that overexpresses BCAR3 under the control of a tet-off promoter under normal culture conditions[7]. As shown in Fig.2C, removal of doxycycline greatly induced BCAR3 expression in these cells (middle panel). Interestingly, TGFβ-induced Smad phosphorylation, observed in the absence of BCAR3, was almost completely blocked when BACAR3 was overexpressed (upper panel). Furthermore, induced BCAR3 expression in MCF-7 cells also impaired Smad nuclear translocation in response to TGFβ (Fig.2D). Together, these results clearly demonstrate that BCAR3 expression is inhibitory to TGFβ-induced Smad phosphorylation and Smad nuclear translocation.

We also investigated the effect of BCAR3 overexpression on TGFβ signaling using fluorescent confocal microscopy. We transiently over expressed FLAG-tagged AND-34 (the mouse homologue of BCAR3) in SUM-159-PT cells, which express low endogenous BCAR3 levels. This allowed us to observe both cells overexpressing BCAR3 and non-overexpressing cells in the same field (Fig.2E). Interestingly, cells expressing FLAG-AND-34/BCAR3 displayed weaker overall phospho-Smad3 signals, compared to non-transfected cells in the same field, under both resting and TGFβ stimulated conditions (Fig.2E). We further quantified Smad3 phosphorylation levels in 5 original confocal images for each experimental condition. As shown in Fig.2F, TGFβ-induced Smad3 phosphorylation was remarkably impaired in cells overexpressing BCAR3, further confirming the results obtained by immunoblot analysis (Fig.2A-D).

3. BCAR3 inhibits TGFβ-mediated Smad transcriptional activity and target gene expression.

We then investigated whether modulating BCAR3 levels could alter Smad-mediated transcriptional activity using a Smad-responsive reporter construct, 12CAGA-lux, which contains 12 repeats of minimal Smad binding site upstream of the firefly luciferase open reading frame. As shown in Fig.3A, TGFβ strongly induced luciferase activity in SCP2 cells transfected with the 12CAGA-lux construct. However, knocking down endogenous BCAR3 using a pool of 2 specific siRNAs against BCAR3 resulted in significant increases in both basal and TGFβ-induced luciferase activity (Fig.3A). Consistently, transient transfection of AND-34 (mouse homologue of BCAR3) into SUM-159-PT cells resulted in significant decreases in both basal and TGFβ-induced luciferase activity (Fig.3B). We further tested, by real-time PCR, whether BCAR3 could alter the expression of *bona fide* TGFβ target genes. Connective tissue growth factor (CTGF)[38], PAI-1[39,40] and Smad7[41] were previously reported to be up-regulated by TGFβ in a Smad-dependent manner. Induced BCAR3 expression in MCF-7 cells almost completely blocked TGFβ's effects to induce CTGF and Smad7, and also remarkably impaired TGFβ's effect to induce PAI-1 expression (Fig. 3C). The difference in effectiveness may be due to involvement of Smad-independent mechanisms downstream of TGFβ. Taken together, these results indicate BCAR3 antagonizes Smad transcriptional activity.

MCF-7 cells are luminal-like, estrogen responsive, and relatively well-differentiated. These cells retain partial cytostatic response to TGFβ. We therefore investigated whether ectopic BCAR3 could antagonize TGFβ's growth inhibitory effects in these cells. Stable MCF-7 cells cultured with doxycycline expressed low level of BCAR3. TGFβ treatment

resulted in 25% reduction in cell viability, as determined by an MTT cell viability assay. Stable BCAR3 expression, on the other hand, reversed TGFβ's effect, resulting in less than 10% reduction in cell viability (Fig.3D). As such, ectopic BCAR3, through inhibition of Smad-mediated transcriptional activity, also inhibits Smad-dependent cytostatic effects.

4. BCAR3 antagonizes TGFβ's pro-migratory and pro-invasive responses.

A hallmark effect of TGFβ in breast cancer cells, particularly in basal-like and triple-negative cells, is single cell migration[17,26,42,43]. Lines of evidence suggest that TGFβ reprograms transcriptional profiles in breast cancer cells to induce epithelial to mesenchymal transition, formation of filopodia, and switching from collective cell migration to single cell migration and ultimately to facilitate intravasation[26,42,44,45]. These effects, although not necessarily concomitant, highlight the pro-migratory role of TGFβ. As such, we investigated whether modulating BCAR3 levels in basal-like breast cancer cells could affect TGFβ-induced cell migration. For this, we silenced endogenous BCAR3 gene in SCP2 cells using 2 specific siRNAs, and examined TGFβ-induced cell migration using the Incucyte time-lapse video imaging migration assay, as previously described[26]. This method couples wound-healing assay with quantitative imaging, and present cell migration by relative wound density, which is the real-time ratio between cell densities within the initial wound area to cell density of adjacent, non-wounded area. As such, this method precludes a net change in cell number over time due to cell proliferation.

TGFβ induced time-dependent migration of SCP2 cells (Fig.4A and Fig.4B). The effect was detectable as early as 12 hrs following stimulation of the cells and further increased

over time to reach a plateau at 48 hours. Cell density in the wounded area is slightly over 52% of that of the adjacent area. TGFβ treatment resulted in an about 10% increase in relative cell density, indicative of more cells in the wounded area. Individual siRNAs against BCAR3 decreased cell migration; marked by only about 42% increase in relative wound density after 48 hours (Fig.4A). This is consistent with previous findings that ectopic BCAR3 expression increases cell migration[9]. Interestingly, cells transfected with the siRNAs displayed an increased response to TGFβ, marked by about 15% increase in relative cell density (Fig. 4A). The 2 siRNA constructs seemed to have similar effects. As such, these data indicate that endogenous BCAR3 is an antagonistic molecule of TGFβ-induced cell migration.

Single cell migration requires actin being organized into treadmilling filaments oriented toward lamilipodia[46]. As such, we investigated whether ectopic BCAR3 expression could antagonize TGFβ's effects on the formation of these structures in SUM-159-PT cells, since these cells express a low level of endogenous BCAR3. Cells were transiently transfected with the FLAG-AND34 (BCAR3) cDNA to allow for the examination of both positive and negative BCAR3 overexpressing cells in the same field. In the absence of BCAR3 overexpression, TGFβ induced a network of elongated actin stress fibers aligned toward filopodia-like structures, indicative a pro-migratory phenotype (Fig.4C, white arrows). In contrast, cells overexpressing BCAR3, although still contained actin filaments, failed to display dominant filopodia-like structures. Rather, they contained relatively short, branched fibers that oriented towards all directions, even when they were stimulated with TGFβ (Fig.4C, yellow arrows). We observed these phenotypes with virtually all transfected cells. Taken together, these data demonstrate that BCAR3 could

antagonize TGFβ's pro-migratory function, likely by interfering with TGFβ-mediated actin filament rearrangement and filopodia formation.

Digestion of extracellular matrix is both a major event during cell invasion and an indication of aggressive property of cancer cells. TGFβ acts as a potent pro-invasive factor in breast cancer and it was previously that this growth factor could increase digestion of a gelatin matrix by MDA-MB-231 cells, using a matrix degradation assay [47]. As such, we investigated whether endogenous BCAR3 could antagonize the TGFβ's pro-invasive effects. As shown in Fig. 5, mock-transfected and control siRNA transfected MDA-MB-231 displayed low of invasive properties under basal conditions. Indeed, when plated on coverslips coated with Alexa488-tagged gelatin, these cells produced small and scattered areas of digestion underneath their bodies, observed as dark spots under confocal microscope (as indicated by the yellow arrows in Fig. 5A). Under these conditions, mock transfected and control siRNA transfected MDA-MB-231 cells treated with TGFβ displayed a clear increase in the total area of digested matrix (Fig. 5A and 5B). Many of these digested areas were elongated, indicative of cell movement during matrix digestion (Fig. 5A). Noticeably, instead of scattering underneath the cell body, elongated digestion spots tend to aggregate at the cell protrusions, overlapping with the lamilipodia-like structures formed by bundled actin filaments (white arrows). These data suggest that in addition to increasing digestion of gelatin matrix, TGFβ also affects the localization of the invadopodia in MDA-MB-231 cells, and remodels their structure from a scattered to an aggregated pattern. Interestingly, transfection of MDA-MB231 cells with a pool of BCAR3 siRNAs significantly potentiated TGFb's effects on matrix digestion, as illustrated by the large areas of digested gelatin (Fig.5A). Particularly, these areas were

formed by multiple elongated spots roughly parallel to each other's. These spots also appeared to be longer than those formed in control cells. Efficiency of the siRNA knockdown was verified by Western blotting, as shown in Fig.5C. Taken together, these results indicate that BCAR3 gene silencing, by means of RNA interference, potentiates TGFβ-induced invadopodia activity and matrix digestion, strongly suggesting that endogenous BCAR3 inhibits TGFβ-induced invadopodia remodeling and matrix digestion.

5. BCAR3 requires p130Cas to antagonize Smad signaling.

Previous studies indicated that the p130Cas physically interacts with Smad2/3 and antagonize Smad activation[33,34]. As p130Cas also interacts with BCAR3, this prompted us to investigate whether p130Cas is involved in BCAR3-mediated inhibition of TGFβ/Smad signaling. Using co-immunoprecipitation analysis in SCP2 cells, we found p130Cas to be constitutively associated with Smad2/3 (Fig.6A). However, knocking down endogenous BCAR3 expression, by means of RNA interference, effectively impaired this association, suggesting that endogenous BCAR3 promotes the interaction between p130Cas and Smad2/3 (Fig.6A).

We next investigated if BCAR3 also required p130Cas to modulate TGFβ-induced cell migration. Transfecting cells with siRNAs targeting BCAR3 and p130Cas both decreased basal cell migration, and increased TGFβ-induced cell migration (not shown and Fig. 6B, respectively). However, upon silencing p130Cas, BCAR3 siRNA lost the effect to further potentiate TGFβ-induced cell migration (Fig. 6C). Indeed, TGFβ stimulation resulted in about 40% increase in cell migration in mock-transfected cells at 36 hours post-stimulation. Transfecting cells with BCAR3 siRNA, or p130Cas siRNA, or both, all

resulted in about 70% increases. Co-transfection of the 2 siRNAs did not have a more-than-additive effect (Fig. 6D), suggesting that BCAR3 requires the presence of p130Cas to antagonize TGFβ function. Altogether, these data suggest that BCAR3 modulates an interaction between p130Cas and Smad2/3, thereby blocking TGFβ /Smad-mediated cell migration.

6. BCAR3 mediates a positive feedback of TGFβ signaling in breast cancer cells.

Despite of extensive studies on the molecular functions of BCAR3, there is no report on how BCAR3 gene expression is regulated. As cellular signaling pathways are often modulated by feedback regulatory loop mechanisms to ensure defined signaling intensity and duration, we investigated whether TGFβ signaling itself could modulate BCAR3 gene expression. For this, we stimulated a panel of breast cancer cell lines with TGFB for 24 hours, and examine the protein level of BCAR3. As shown in Fig.7A, TGFβ treatment resulted in a remarkable decrease in BCAR3 protein levels in all cell line tested, highlighting BCAR3 as a novel target for TGFβ signaling. Furthermore, our results also indicate that TGFβ-mediated suppression of BCAR3 gene expression depends on the canonical Smad2/3 signaling, as silencing either Smad2 or Smad3 gene expression in BT-549 cells using specific siRNAs in BT-549 cells almost completely abolished TGFβ's effect in decreasing BCAR3 protein expression (Fig. 7B). Taken together, our results define a positive feedback loop mechanism, by which TGFβ/Smad signaling transcriptionally repress expression of its own inhibitory molecule, BCAR3, further leading to enhanced TGFβ/Smad signaling in breast cancer cells (Fig. 7C).

Discussion:

In this study, we defined a novel role for BCAR3 to antagonize the canonical TGFβ signaling by promoting an interaction between p130Cas and the Smad transcription factors. The inhibitory effect of BCAR3 on TGFβ/Smad signaling is observed in all breast cancer cell lines tested, regardless of their molecular phenotype and biological responses to TGFβ. While TGFβ efficiently induces growth arrest in normal mammary epithelial cells and early breast carcinoma, these cytostatic responses are lost in more advanced, invasive breast tumors and replaced by TGFβ-induced tumor promoting and pro-metastatic responses [48,49]. In invasive, basal-like breast cancer cells, such as MDA-MB-231, SCP2 and SUM-159-PT, BCAR3 potently antagonizes TGFβ's effects on cell migration, formation of filopodia-like structures and digestion of gelatin matrix. On the other hand, in the luminal-like, ER+ MCF7 cells, BCAR3 overexpression antagonized TGFβ's cytostatic effect. These findings imply that the novel function of BCAR3 to inhibit Smad signaling is likely a conserved mechanism among the different molecular subtypes of breast cancer.

Interestingly, we found BCAR3 to be a novel target gene of TGFβ in breast cancer cells. TGFβ decreased BCAR3 protein expression in multiple breast cancer cells. This effect appeared to be Smad-dependent, as it could be abolished by knocking down either Smad2 or Smad3. These data are the first to describe a molecular mechanism by which BCAR3 expression is being regulated. More importantly, our data define a positive feedback mechanism downstream of TGFβ/Smad signaling. It is known that a few negative feedback loops exist to fine-tune TGFβ signaling, such as those mediated by Smad7[41] and GRK2[50]. Both of these targets were shown to be being up-regulated by TGFβ, further leading to termination of Smad signaling[41,50,51]. To our knowledge, BCAR3 serves as the

first example of a positive feedback loop of TGFβ/Smad signaling, whereby TGFβ signaling itself can decrease the expression levels of its inhibitor BCAR3, further leading to enhanced and potentiated TGFβ/Smad signaling. Such a mechanism may be important for maintaining a steady response to TGFβ.

It has been established that active Smad signaling contributes to breast cancer local invasion and distant metastasis. Having defined BCAR3 as a novel TGFβ/Smad inhibitory molecule, this may account for the low BCAR3 expression levels observed in primary breast tumors associated with worse prognosis and higher chances of disease progression. Indeed, low BCAR3 levels may release tumor cells from its inhibitory effect on Smad signaling, leading to enhanced, more potent TGFβ activity and tumor progression. Results from our clinical data survey and analysis define BCAR3 as a single factor whose expression level is predictive of clinical outcomes in breast cancer patients. Moreover, we also found that patients with lymph node metastasis tend to carry loss of heterozygosity at BCAR3 alleles, indicating that BCAR3 has some features of a tumor suppressor-like gene to prevent disease progression.

Conclusion:

Taken together, our study identified a novel positive feedback loop mechanism downstream of the canonical TGFβ/Smad signaling axis, mediated by a breast cancer anti-estrogen resistance gene, BCAR3. We report a novel role of BCAR3 to antagonize Smad signaling, efficiently leading to inhibition of the TGFβ's biological functions in breast cancer cells. Our results also highlighted a true prognostic value for BCAR3 in human breast cancer, as we found low BCAR3 expression levels in primary breast tumors correlates with poor outcomes regardless of treatment plans. Our study provides new insights into BCAR3's mechanism of action, and suggests a need to re-evaluate BCAR3's implications in breast cancer pathology.

Abbreviations:

BCAR3: Breast Cancer Anti-Estrogen Resistance 3; DMEM: Dulbecco's modified Eagle's medium; DMFS: distant metastasis free survival; ER: estrogen receptor; FBS: fetal bovine serum; MTT: thiazolyl blue tetrazolium bromide; OS: overall survival; RFS: relapse free survival; TGFβ: transforming growth factor-beta.

Competing interests:

The authors declare no competing interests.

Author contribution:

JG and JJL designed the experiments, involved in all experiment procedures, analysis and interpretation, and wrote the manuscript. JK, MD and NFA performed some of the experiments. CVR and MP assisted in designing matrix degradation assays. SA assisted in designing experiments and interpreting results.

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Figure legends:

Figure 1. Low BCAR3 expression predict poor prognosis in human breast cancer.

A and B. Kaplan-Meier survival curved generated by GOBO gene expression-based outcome tool, showing status of progression free survival (PFS) of a compiled cohort of breast cancer patients who underwent various treatment plans (A) and a compiled cohort of patients who received no systematic treatment (B). Patients were separated by median of signal intensity from an Affymetrix probe targeting BCAR3 (204032_at) in microarray analysis. Survival data of the high expression group was shown by the red curve, and that of the low expression group was shown by the gray curve. **C and D.** Kaplan-Meier survival curves showing status of distant metastasis free survival (DMFS) of a compiled cohort of breast cancer patients who underwent various treatment plans (C) and a compiled cohort of patients who received no systematic treatment (D). Patients were separated by median of signal intensity from 204032_at. **E and F.** Kaplan-Meier survival curves showing status of relapse free survival (RFS) of a compiled cohort of breast cancer patients who underwent various treatment plans (E) and a compiled cohort of patients who received no systematic treatment (F). Patients were separated by median (E) or into 5 groups (F). **G.** Dot plot of reading of BCAR3 extracted from the NCBI-GEO dataset GDS-807, which includes microarray readings of gene expression in micro-dissected primary tumors from patients who subsequently received Tamoxifen treatment for 5 years. **H.** Percentage of loss of heterozygosity at BCAR3 alleles in breast tumors with increasing N-stage.

Figure 2. BCAR3 antagonizes Smad activation.

A. Total cell lysates from breast cancer cells were subjected for Western blot analysis for expression of BCAR3 and p130Cas. **B.** SCP2 cells were transfected with 50pM BCAR3

siRNA, starved overnight and stimulated with 100pM TGFβ at 48 hours post-starvation for time periods as indicated. Levels of phospho-Smad3 and BCAR3 were examined by Western blot. **C.** Inducible MCF-7 cells were cultured with or without 1□g/ml doxycycline, starved overnight and stimulated with 100pM TGFβ at 96 hours post-treatment for time periods as indicated. Levels of phospho-Smad3 and BCAR3 were examined by Western blot. **D.** The stable MCF-7 cells were treated with doxycycline and stimulated as described. Levels of phosphor-Smad3 in nuclear extracts were examined by Western blot. **E.** SUM-159-PT cells were seeded onto glass coverslips, transfected with FLAG-tagged AND-34 (mouse homologue of BCAR3) and starved overnight. Cells were then treated with or without 200pM TGFβ and subjected for immunofluorescence microscopy and DAPI counter staining. In the merged images, phosphor-Smad3 is shown in red, FLAG-AND-34 is shown in green and cell nucleuses are shown in blue. **F.** phosphor-Smad3 signal in transfected and non-transfected cells were quantified from 5 original LSM images. Error bars show standard errors of the mean. An asterisk indicates a statistical difference between the two groups compared, as determined by unpaired Student's T-test ($p<0.05$).

Figure 3. BCAR3 antagonizes function of canonical TGFβ signaling.

A. SCP2 cells were co-transfected with 12CAGA-lux luciferase reporter construct, constitutive β-gal construct and 50pM scrambled siRNA control, or a pool of siRNAs targeting BCAR3 (25pM each), or 50pM individual siRNA targeting BCAR3. Cells were starved overnight, stimulated with or without 100pM TGFβ and subjected for luciferase assays. Luciferase activities were normalized by β-gal activity, and represented as relative luciferase units (RLUs). Error bars show standard errors of the mean of 3

independent experiments. An asterisk indicates a statistical difference, as determined by unpaired Student's T-test ($p<0.05$). **B.** SUM-159-PT cells were co-transfected with 12CAGA-lux luciferase reporter construct, constitutive β-gal construct and FLAG-AND-34. Luciferase assays were performed as described above. **C.** Inducible MCF-7 cells were cultured with or without doxycycline for 72 hours, starved overnight and stimulated with 100pM TGFβ for 24 hours. mRNA levels of 3 Smad-dependent genes were examined by real-time PCR. Error bars show SEM of 3 independent experiments. **D.** Inducible MCF-7 cells were cultured with or without doxycycline for 72 hours, starved overnight and stimulated with 100pM TGFβ for 72 hours. Cell viability was examined by an MTT assay. Error bars show SEM of 3 independent experiments. An asterisk indicates a statistical difference.

Figure 4. Knocking down BCAR3 promotes TGFβ-induced cell migration.

A. SCP2 cells were transfected, scratched and treated as described in Materials and Methods. Real-time migration profiles were compared for mock transfected cells and cells transfected with two different BCAR3 siRNAs. Error bars show standard errors of 4 biological replicates. **B.** representative figures of cell migration from initial scratch (bounded by black regions). Migrated cells were shown by gray zones. **C.** SUM-159-PT cells were transfected with FLAG-AND-34, starved and treated with or without TGFβ, then subjected for immunofluorescent microscopy. In the merged images, FLAG-AND-34 is shown in green, F-actin is shown in red and cell nucleuses are shown in blue.

Figure 5. Knocking down BCAR3 promotes TGFβ-induced digestion of gelatin matrix.

A. SCP2 cells were transfected with scrambled control siRNA, or BCAR3 siRNA, or mock transfected for 48 hours. Cells were then seeded onto coverslips coated with Alexa488-conjugated gelatin, treated with or without TGFβ and allowed to digest the

matrix for 36 hours. Representative images show immunofluorescence staining of BCAR3 (white), Actin filaments (red) and gelatin matrix (green). Results represent 3 independent experiments. **B.** The total areas of digestion (pixel count) were quantified for 10 cells transfected with scrambled siRNA, or BCAR3 siRNA, or mock transfected. Cells were from at least 5 LSM images. Error bars showed SEM. **C.** Western blot showing the effect of BCAR3 siRNA. Total cell lysates were collected from the cells from the same pools as the ones seeded onto cover slips.

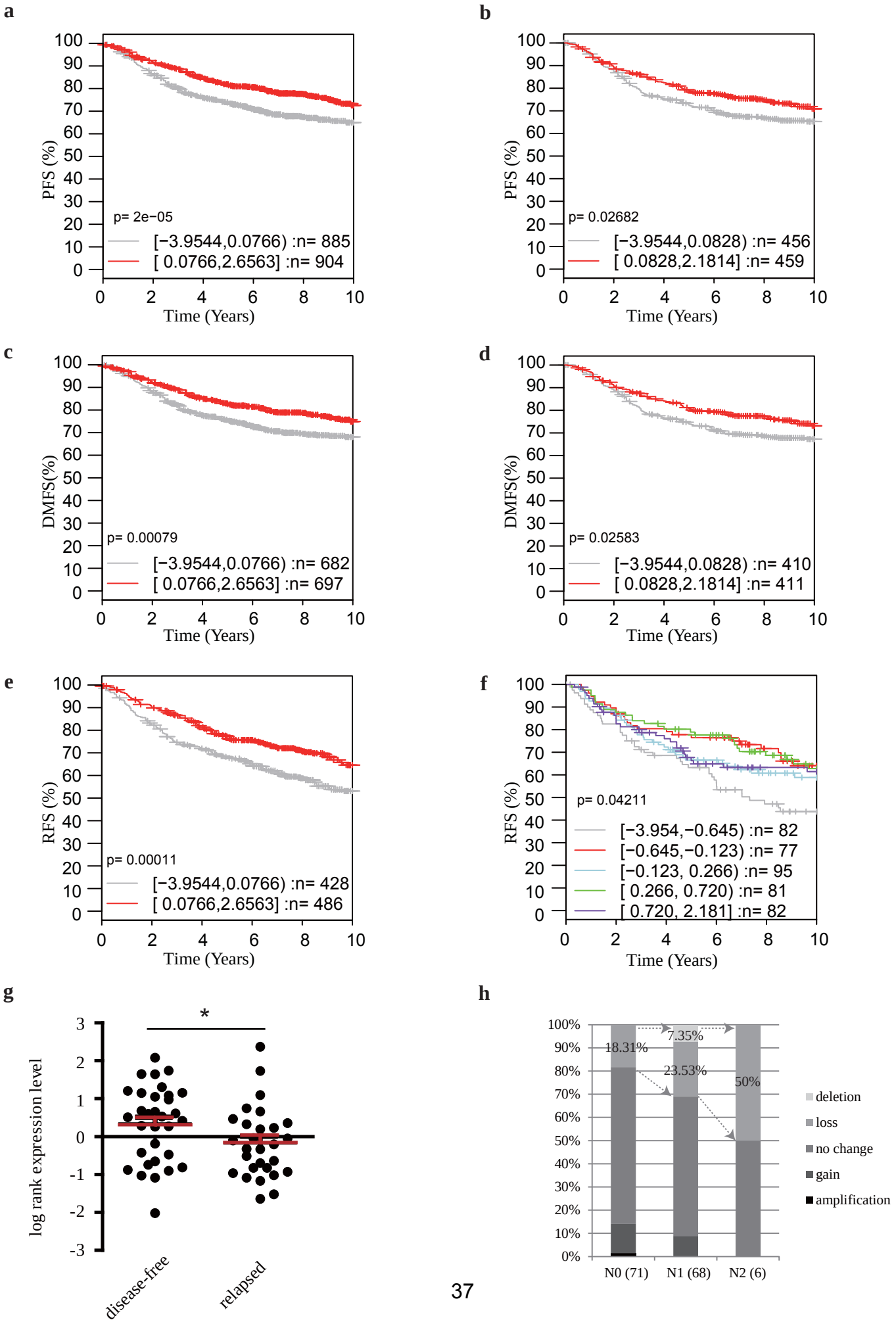
Figure 6. BCAR3 requires p130Cas to antagonize TGFβ signaling.

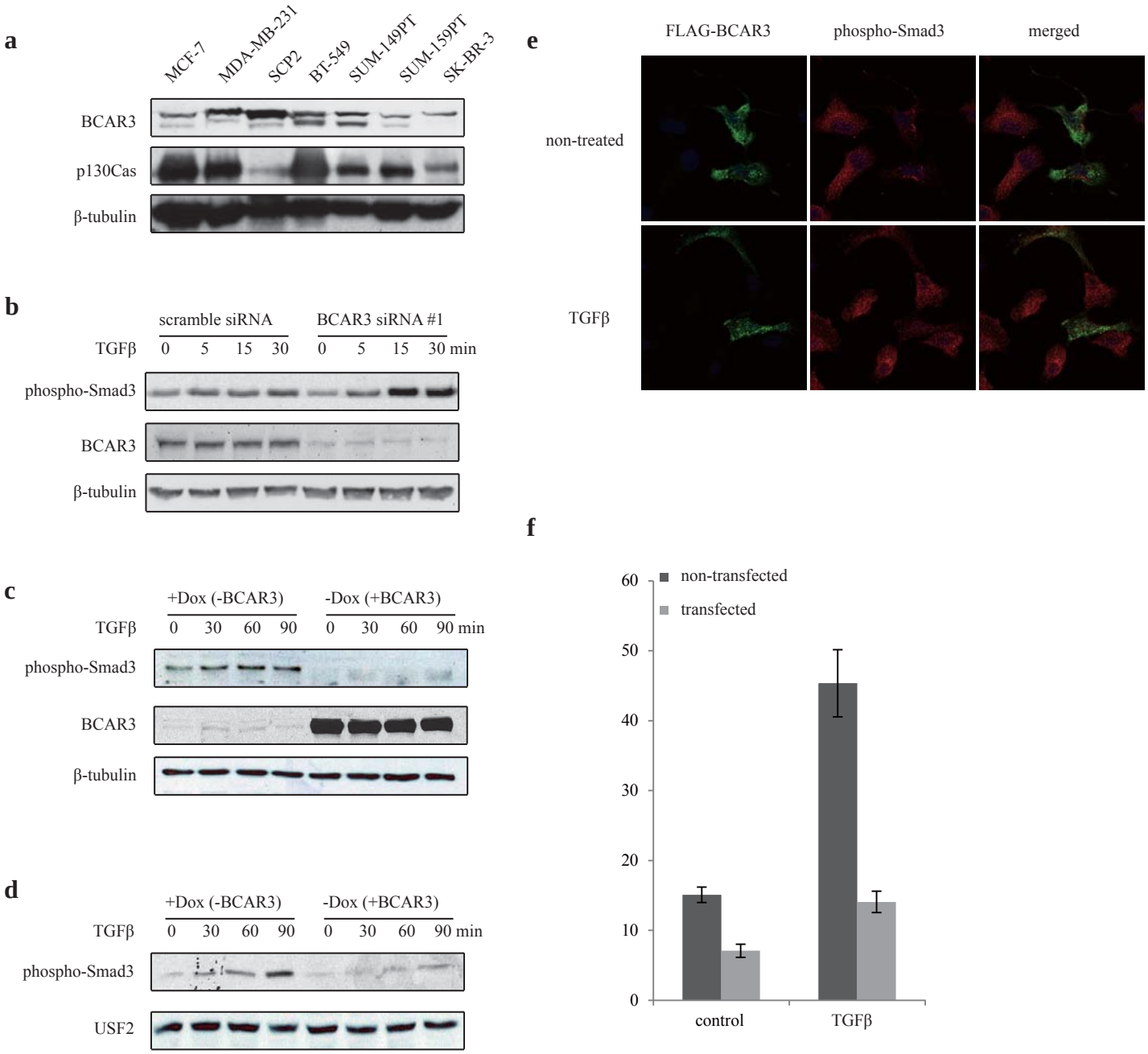
A. SCP2 cells were transfected with BCAR3 siRNA, starved overnight and stimulated with TGFβ. Smad2/3 were immunoprecipitated and levels of p130Cas in the precipitant were examined by Western blot. **B.** SCP2 cells were transfected, starved then stimulated with TGFβ. Levels of phospho-Smad3 and BCAR3 were examined by Western blot. Signal densities of phospho-Smad3 were normalized with that of total Smad3, and shown below the top panel. **C.** SCP2 cells were transfected with BCAR3 siRNA or p130Cas siRNA or together, starved, stimulated and subjected for Incucyte cell migration assays. Real-time migration profiles were compared for mock transfected cells and cells transfected with BCAR3 siRNA (C), p130Cas siRNA (D) and for cells transfected with p130Cas siRNA and both siRNAs (E). Error bars show standard deviation of the mean for 6 biological replicates. F. Relative wound densities at 36h are plotted. Error bars show standard deviation of the mean for 6 biological replicates.

Figure 7. TGFβ down-regulates BCAR3 in a Smad-dependent manner.

A. Breast cancer cells were starved and treated with TGFβ for 24 hours. Levels of BCAR3 protein expression were examined by Western blot. **B.** BT-549 cells were

transfected with Smad2 siRNA or Smad3 siRNA for 48 hours, starved and treated with TGFβ for 24 hours. Levels of BCAR3 protein expression were examined by Western blot. **C.** Model of BCAR3 in mediating a positive feedback loop downstream of TGFβ/Smad signaling axis.


a
PFS (%)
p= 2e−05
[−3.9544,0.0766) :n= 885
[0.0766,2.6563] :n= 904
Time (Years)
b
PFS (%)
p= 0.02682
[−3.9544,0.0828) :n= 456
[0.0828,2.1814] :n= 459
Time (Years)
c
DMFS(%)
p= 0.00079
[−3.9544,0.0766) :n= 682
[0.0766,2.6563] :n= 697
Time (Years)
d
DMFS(%)
p= 0.02583
[−3.9544,0.0828) :n= 410
[0.0828,2.1814] :n= 411
Time (Years)
e
RFS (%)
p= 0.00011
[−3.9544,0.0766) :n= 428
[0.0766,2.6563] :n= 486
Time (Years)
f
RFS (%)
p= 0.04211
[−3.954,−0.645) :n= 82
[−0.645,−0.123) :n= 77
[−0.123, 0.266) :n= 95
[0.266, 0.720) :n= 81
[0.720, 2.181] :n= 82
Time (Years)
g
log rank expression level
*
disease-free
relapsed
h
18.31%
7.35%
23.53%
50%
N0 (71)
N1 (68)
N2 (6)
deletion
loss
no change
gain
amplification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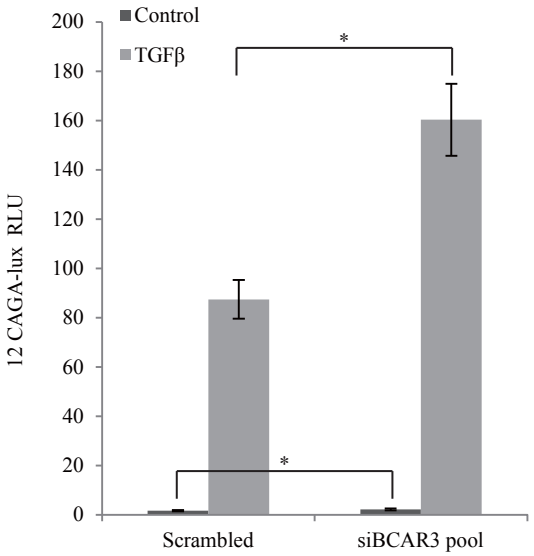
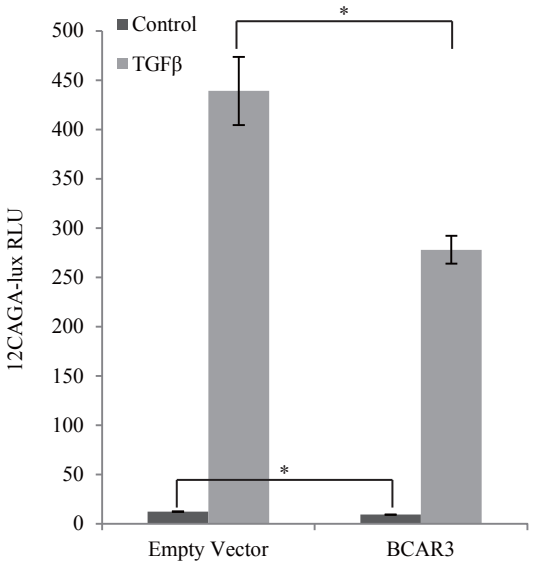
MCF-7
MDA-MB-231
SCP2
BT-549
SUM-149PT
SUM-159PT
SK-BR-3
BCAR3
p130Cas
β-tubulin
b
scramble siRNA
BCAR3 siRNA #1
TGFβ 0 5 15 30 0 5 15 30 min
phospho-Smad3
BCAR3
β-tubulin
c
+Dox (-BCAR3)
-Dox (+BCAR3)
TGFβ 0 30 60 90 0 30 60 90 min
phospho-Smad3
BCAR3
β-tubulin
d
+Dox (-BCAR3)
-Dox (+BCAR3)
TGFβ 0 30 60 90 0 30 60 90 min
phospho-Smad3
USF2
e
FLAG-BCAR3
phospho-Smad3
merged
non-treated
TGFβ
f
non-transfected
transfected
60
50
40
30
20
10
0
control
TGFβ

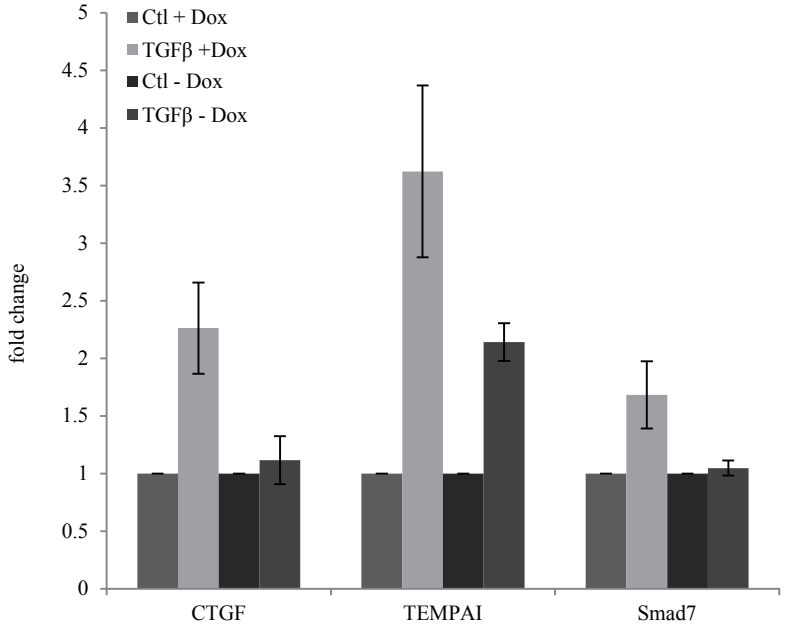
a


Control
TGFβ
12 CAGA-lux RLU
200
180
160
140
120
100
80
60
40
20
0
*
*
Scrambled
siBCAR3 pool

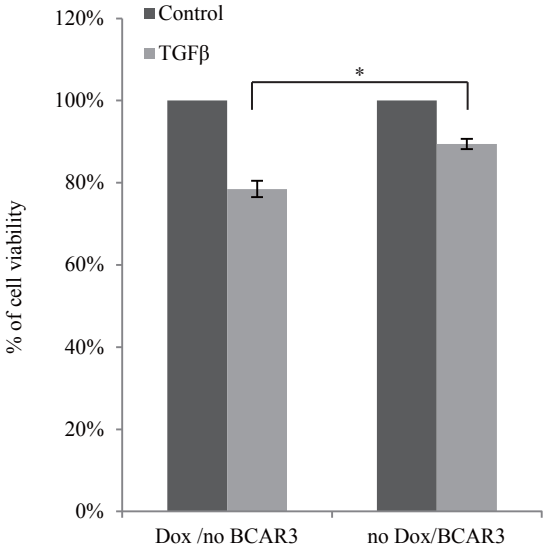
b

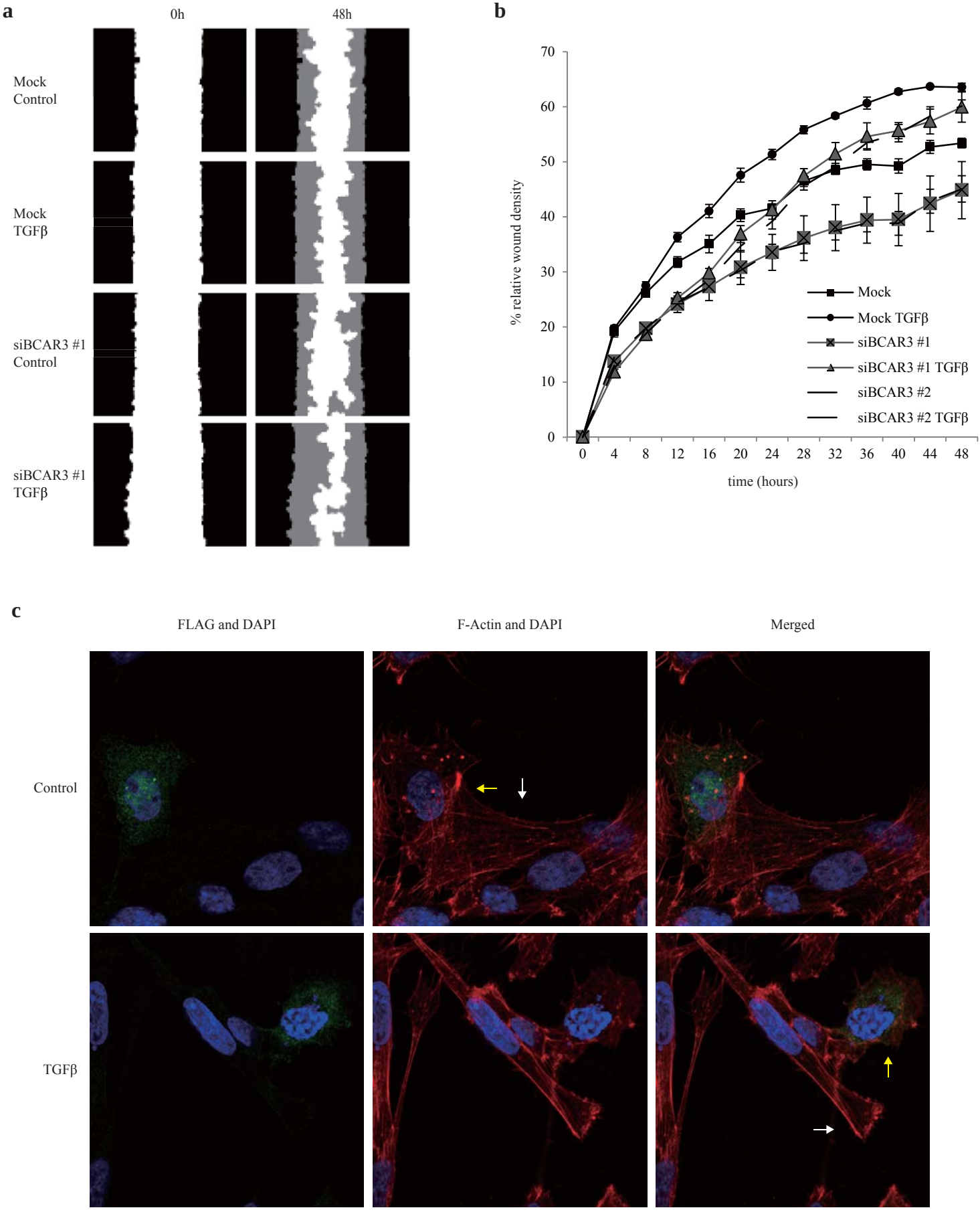

Control
TGFβ
12CAGA-lux RLU
500
450
400
350
300
250
200
150
100
50
0
*
*
Empty Vector
BCAR3

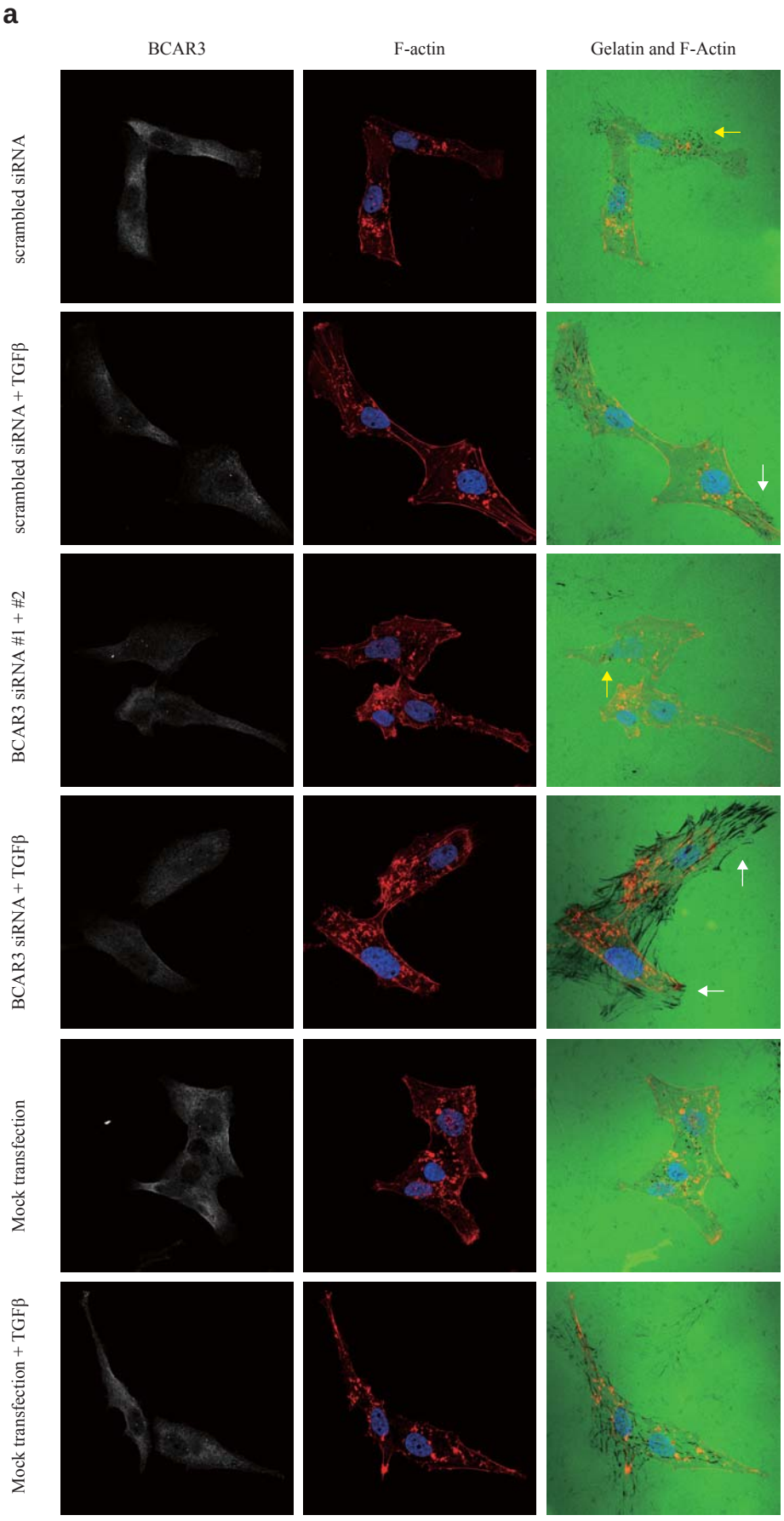
c

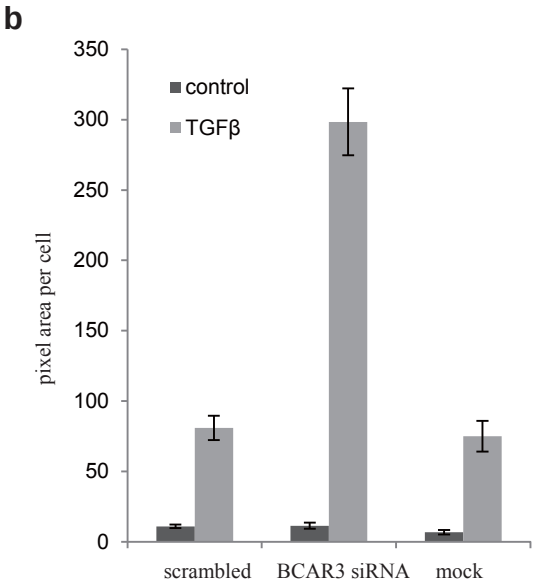

Ctl + Dox
TGFβ +Dox
Ctl - Dox
TGFβ - Dox
fold change
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
CTGF
TEMPAI
Smad7

d


Control
TGFβ
% of cell viability
120%
100%
80%
60%
40%
20%
0%
*
Dox /no BCAR3
no Dox/BCAR3


a
0h
48h
Mock Control
Mock TGFβ
siBCAR3 #1 Control
siBCAR3 #1 TGFβ
b
% relative wound density
time (hours)
Mock
Mock TGFβ
siBCAR3 #1
siBCAR3 #1 TGFβ
siBCAR3 #2
siBCAR3 #2 TGFβ
c
FLAG and DAPI
F-Actin and DAPI
Merged
Control
TGFβ


a
BCAR3
F-actin
Gelatin and F-Actin
scrambled siRNA
scrambled siRNA + TGFβ
BCAR3 siRNA #1 + #2
BCAR3 siRNA + TGFβ
Mock transfection
Mock transfection + TGFβ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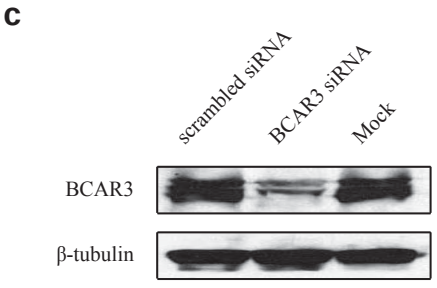
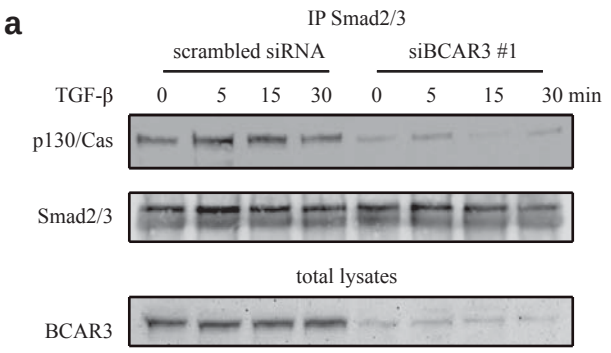
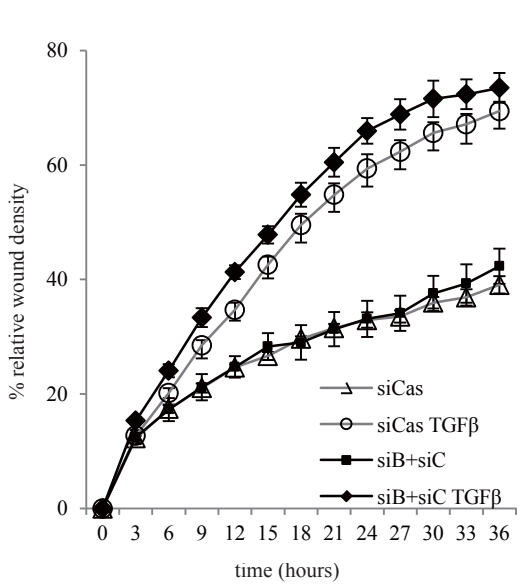
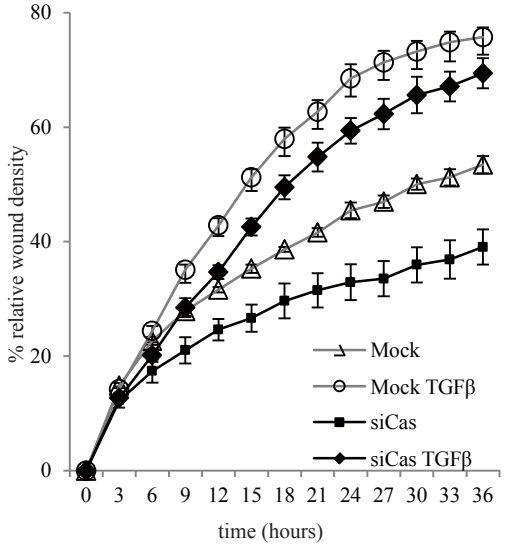
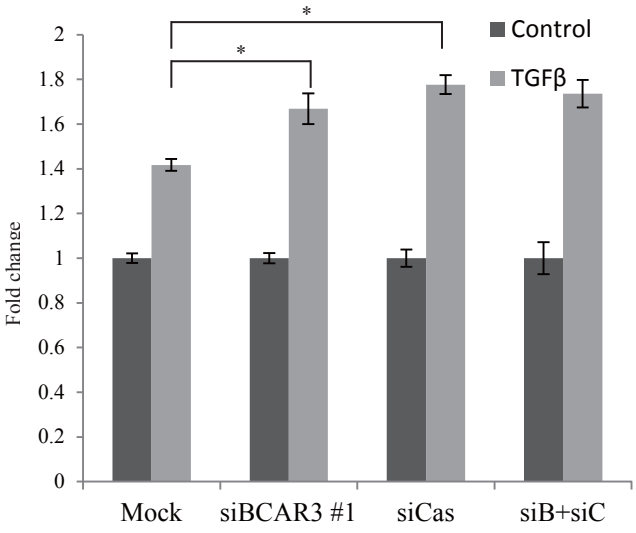
350
300
250
200
150
100
50
0
control
TGFβ
pixel area per cell
scrambled
BCAR3 siRNA
mock

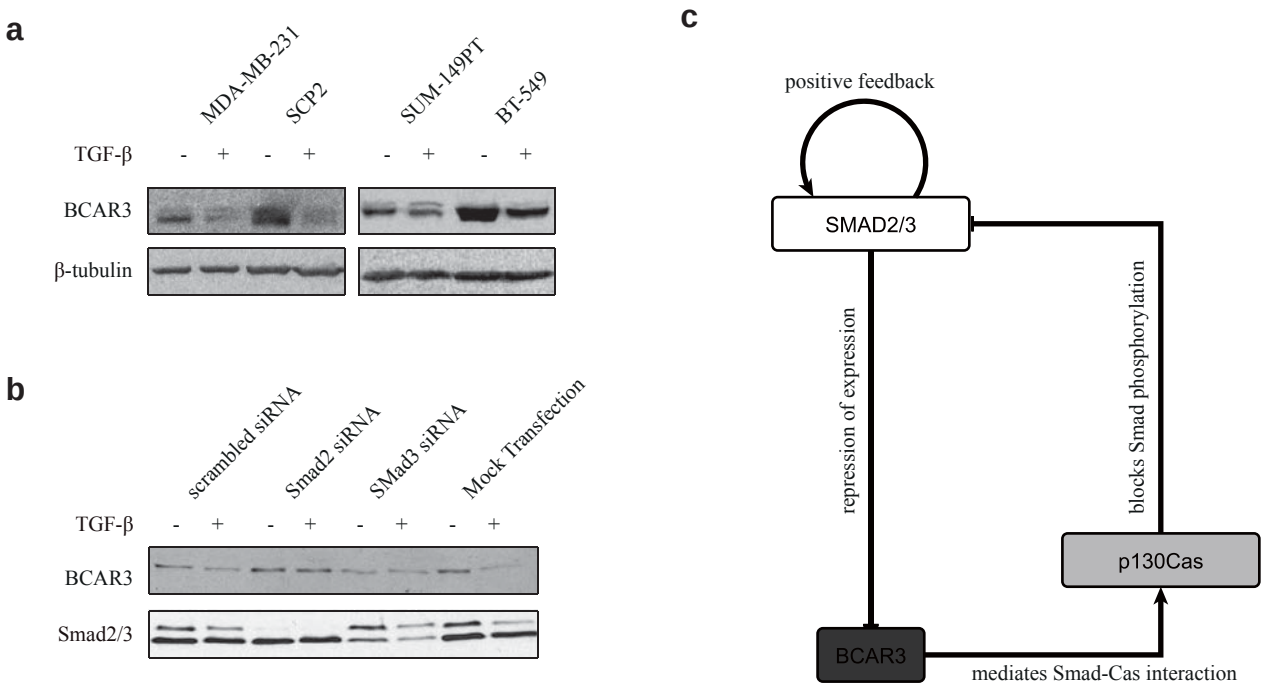

c
scrambled siRNA
BCAR3 siRNA
Mock
BCAR3
β-tubulin


a
IP Smad2/3
scrambled siRNA
siBCAR3 #1
TGF-β
0
5
15
30
0
5
15
30 min
p130/Cas
Smad2/3
total lysates
BCAR3


c
% relative wound density
time (hours)
siCas
siCas TGFβ
siB+siC
siB+siC TGFβ


b
% relative wound density
time (hours)
Mock
Mock TGFβ
siCas
siCas TGFβ


d
Fold change
Control
TGFβ
Mock
siBCAR3 #1
siCas
siB+siC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MDA-MB-231
SCP2
SUM-149PT
BT-549
TGF-β
- + - + - + - +
BCAR3
β-tubulin
b
scrambled siRNA
Smad2 siRNA
SMad3 siRNA
Mock Transfection
TGF-β
- + - + - + - +
BCAR3
Smad2/3
c
positive feedback
SMAD2/3
repression of expression
blocks Smad phosphorylation
p130Cas
BCAR3
mediates Smad-Cas interaction

Appendix B:

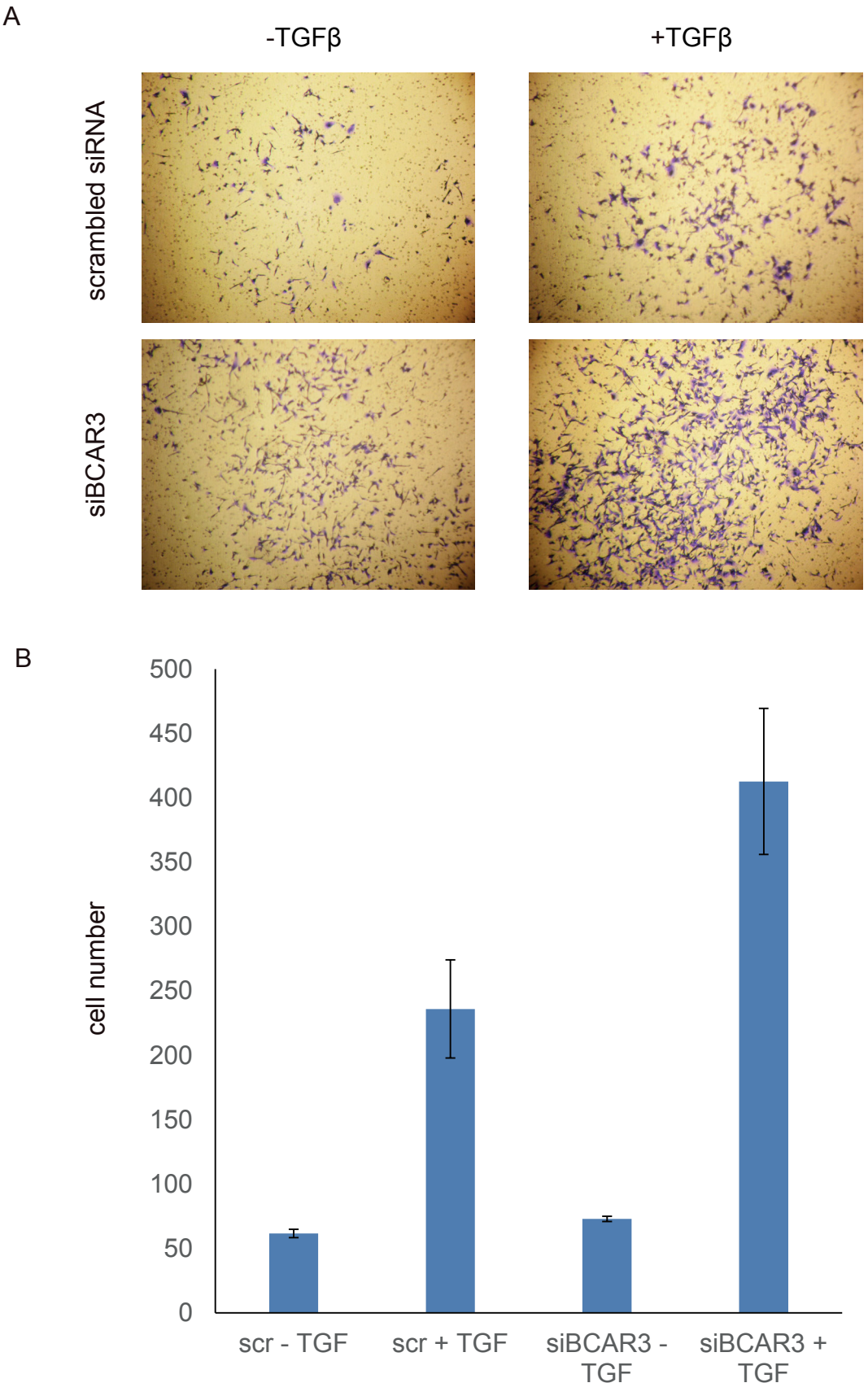

Figure 1. Knocking down BCAR3 enhances TGFβ-induced cell invasion through matrigel.
A. SCP2 cells were transfected with scrambled siRNA or siRNAs targeting BCAR3 for 72 hours. Cells were then stimulated with or without 100 pM TGFβ, and subjected for transwell invasion assays for 24 hours. Images of the centers of each transwell chamber were taken. B. Quantification of numbers of invaded cells of a biological triplicate of one representitive experiment. Figures represent results of 4 independent experiments.

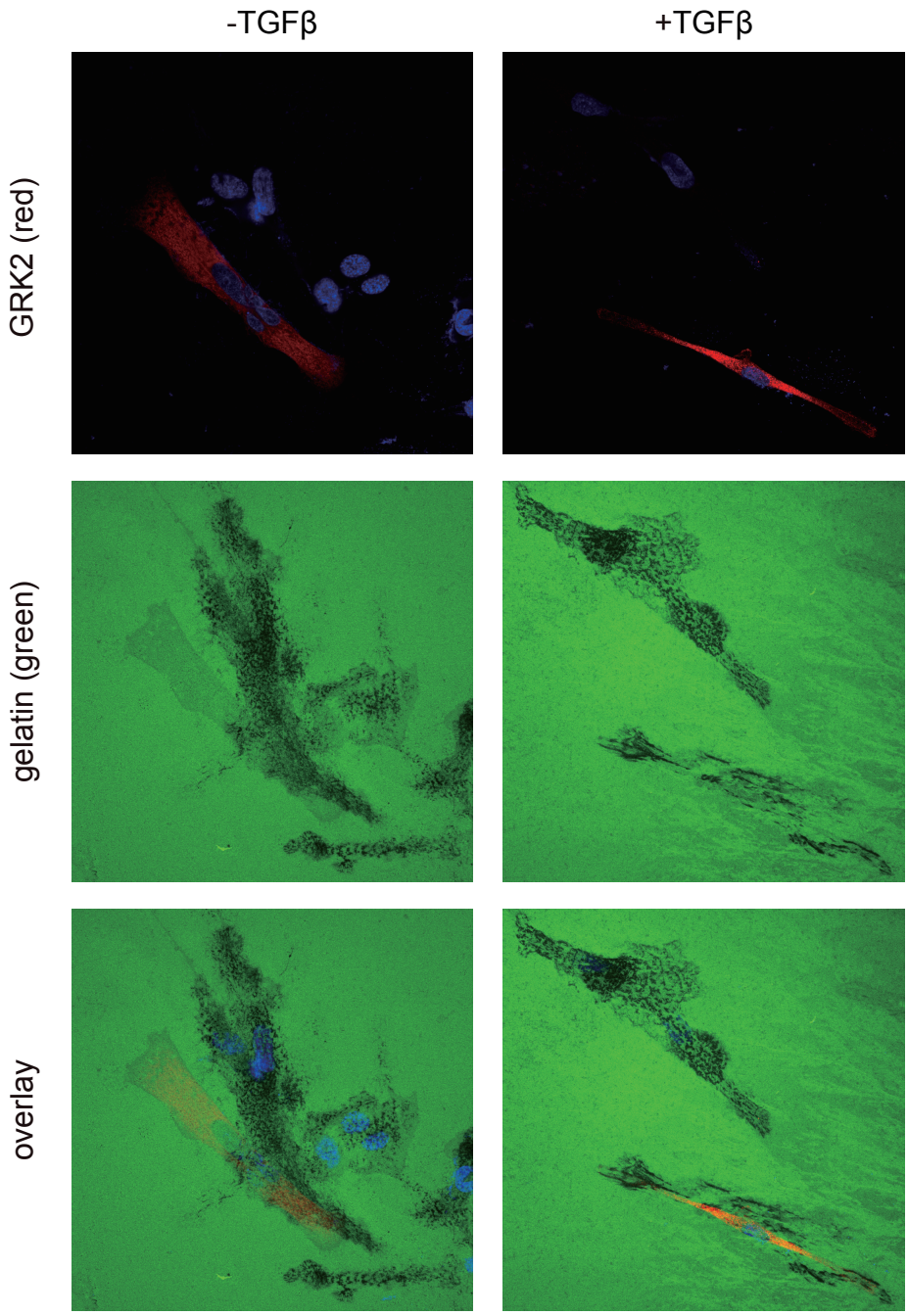

Figure 2. GRK2 overexpression decreases TGFβ-induced matrix degradation.
MDA-MB-231 cells were transfected with FLAG-tagged GRK2 and seeded on coverslips coated with Alexa488-labeled gelatin matrix. Cells were then stimulated with or without TGFβ for 36h, and subjected for immunofluorescence microscopy analysis.

Appendix C:

Publications:

4. **Guo J**, Korah J, Rajadurai CV, Dai M, Fils-Aime N, Park M, Ali S, L and Lebrun J-J, Breast Cancer Anti-Estrogen Resistance-3 inhibits TGFβ/Smad signaling and associates with favorable breast cancer disease outcomes. (under review)

3. Benyoucef Y, Shams A, Liu F, Korah J, **Guo J**, Garic D, Dai M, Yasruel Z, Lebrun JJ and Ali S, A Protective Role for Prolactin in Human Breast Cancer Reveals Novel Function for the RNA-Binding Protein Nudt21 and Paraspeckles in Carcinogenesis. (under review)

2. Dai M, Al-Odaini AA, Fils-Aime N, Villatoro MA, **Guo J**, Arakelian A, Rabbani SA, Ali S, Lebrun JJ. Cyclin D1 cooperates with p21 to regulate TGFβ-mediated breast cancer cell migration and tumor local invasion. *Breast Cancer Res.* 2013 Jun 20;15(3):R49.

1. Fils-Aime N, Dai M, **Guo J**, Kahramangil B, Neel JC, Lebrun JJ. Micro-RNA-584 and PHACTR1: New Signaling Route Through Which Transforming Growth Factor-Beta Mediates the Migration and Actin Dynamics of Breast Cancer Cells. *Journal of Biological Chemistry* 2013 Epub.

Posters:

4. **Guo J** and Lebrun JJ, Breast Cancer Anti-Estrogen Resistance 3 Antagonizes Transforming
Growth Factor β signaling and is Correlated to Favorable Disease Outcomes in Breast Cancer Patients. AACR Conference on Mechanism of Resistance to Molecular Targeted Therapies, San Diego CA, USA, May 9-12 2012.

3. **Guo J**, Chen H, Ho J and Lebrun JJ, GRK2 Antagonizes the Pro-migratory TGFβand Pro-angiogenic Ang II Signaling. AACR Conference on Tumor Microenvironment Complexity, Orlando FL, USA, November 3-6 2011.

2. **Guo J** and Lebrun JJ, BCAR3 promotes interaction between p130Cas and Smad3 to antagonize TGFβ induced cell invasion in triple negative breast cancer cells. The Biology of Cancer: Microenvirioment, Metastasis & Therapeutics, Cold Spring Harbor NY, USA, April 26-20 2011.

1. **Guo J** and Lebrun JJ, BCAR3 is down-regulated by TGFβ and provides a positive feedback to TGFβ signaling. McGill University Health Centre Cancer Research Axis Research Day, 2011, Montreal QC, CANADA, Poster 02.